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Combination of Microneutralization and Avidity Assays: Improved Diagnosis of Recent Primary Human Cytomegalovirus Infection in Single Serum Sample of Second Trimester Pregnancy

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Estimation of IgG avidity index is a classical serological method. Antibodies with low avidity are detectable at a very early stage of infection whereas high avidity antibodies indicate past infection. Recently, it was shown that the neutralization assay can be routinely used as a reliable method for differentiating between acute primary and non-primary infection in a single serum sample because the first neutralizing titers (NT) appeared after an average of 13 weeks (range, 10–17 weeks). A low positive NT titer in the presence of specific IgM antibodies, however, still represents a diagnostic problem especially if blood sampling occurred after the 12th week of gestation. To overcome this problem the combination of NT and IgG avidity tests was evaluated. Human cytomegalovirus (HCMV) IgG avidity indices of 350 serum samples from 227 pregnant women were investigated using 6M urea in the washing buffer. HCMV specific IgG antibodies reached full maturation approximately 20–22 weeks after seroconversion and low IgG avidity is therefore a marker of primary infection. The combined application of the microneutralization and avidity assays was shown to serve as a helpful tool in diagnosis of a recent primary HCMV infection of second trimester pregnancy particularly when previous serological data were not available. ***J. Med. Virol. 60: 324–330, 2000.***

KEY WORDS: HCMV; avidity index; neutralizing antibodies; primary infection in pregnancy

INTRODUCTION

Human cytomegalovirus is the most common cause of congenital infection worldwide. Fetal damage is associated most frequently with maternal primary infection as opposed to recurrent infection [Nelson and Demmler, 1997]. As maternal infection is often asymptomatic, diagnosis of acute HCMV infection in pregnancy is based on serological methods, i.e., documented seroconversion or follow-up sera with declining IgM titers. The presence of specific IgM antibodies alone cannot be used to identify primary HCMV infection because specific IgM responses may persist for long periods and may also occur after HCMV reactivation or reinfection [Daiminger et al., 1999]. Recently, we have shown that the neutralization assay can be used routinely as a reliable method for differentiating between acute primary and nonprimary infection in a single serum sample [Eggers et al., 1998]. After seroconversion, the first neutralizing (NT) titers appeared after an average of 15 weeks (range, 10–17 weeks), tended to be low initially (1:4–1:32) and were seen to rise in follow-up sera from 1:64–1:512. A low positive NT titer in the presence of specific IgM antibodies, however, still represents a diagnostic problem especially if blood sampling occurred after the 12th week of gestation. To overcome this problem the combination of microneutralization test and another serological method was evaluated that has been shown to be useful for identifying primary HCMV infection: the IgG avidity test [Blackburn et al., 1991; Lazzarotto et al., 1997; Grangeot-Keros et al., 1997; Bodeus et al., 1998; Bodeus and Goubau, 1999; Lazzarotto et al., 1999]. In the present study the maturation kinetics of CMV-specific IgG avidity after IgG or NT seroconversion were investigated as well as the capacity of the avidity assay to differentiate primary from nonprimary infection in a single serum taken from women during the second trimester of pregnancy.

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MATERIALS AND METHODS

Clinical Samples

A total of 350 sera obtained from 227 pregnant women at various stages of pregnancy were collected from April 1998 until January 1999.

In Group I follow-up sera (n = 89) from 27 women with acute primary infection were investigated. Two to six consecutive blood samples were taken from each woman at intervals of at least two weeks and infection confirmed as primary in each case by documented HCMV IgG seroconversion or by using a microneutralization test (Eggers et al., 1998), based on absence of neutralizing antibodies in the earliest serum sample.

Group II comprised 91 sera from 87 previously infected women that were negative for HCMV-IgM antibodies.

Group III consisted of 170 samples from 113 pregnant women that could not be readily differentiated using standard serological methods because of a positive IgM response in at least one of two tests and positive NT titer.

All samples were collected and stored at −20°C before testing.

Serological Assays

Detection of HCMV-specific IgG antibodies. IgG antibodies in sera were determined using the Enzygnost Anti-HCMV/IgG EIA (Dade Behring, Marburg, Germany) carried out with the Behring ELISA Processor (BEPIII) (Dade Behring, Marburg, Germany) according to the manufacturer's recommendations.

IgG values were expressed in Paul Ehrlich Institute (PEI) units/l (PEI, Langen Germany). Antibody negativity was defined by levels <180 PEI units/l. Sera with an antibody level ≥30.000 PEI units/l were considered to be elevated.

Detection of HCMV specific IgM antibodies.

IgM enzyme linked antigen assay (ELA). HCMV-specific IgM antibodies were detected using an in-house modification of the HCMV IgM ELA (Medac, Hamburg, Germany) a two-step μ-capture EIA. Microtiter plates (Nunc, Denmark) were coated with monoclonal anti-human IgM antibody at a concentration of 1 μg/ml (DAKO, Hamburg, Germany). Serum specimen and calibrators were incubated for 2 hours at room temperature with the solid phase of the microtiter plates. Subsequently, bound anti-HCMV IgM human antibodies were labeled during the second incubation for 2 hours at room temperature with HCMV antigen (strain AD 169) coupled to horseradish peroxidase. The tests were run on the BEP III using washing buffers from the Enzygnost Anti-HCMV/IgM kits (Dade Behring, Marburg, Germany). Titers were defined as follows: <1:32/1:32 = negative, 1:64–1:256 = low positive, 1:512–1:2048 = positive and ≥1: 4096 = high positive.

IgM sorin. The ETI-CYTOK-M reverse μ-capture EIA (Sorin Biomedica, Saluggia, Italy) was carried out according to the manufacturer's recommendations using sera diluted 1:101.

Microneutralization Test

The microneutralization assay was carried out without complement enhancement as described recently by Eggers et al., [1998]. Human embryonic lung fibroblasts (passages 14–17) were seeded in 96-well microtiter plates (2×10^4cells/well) one day before testing. All serum-samples were heat-inactivated (56°C/30 min). Fifty μl of serum (dilutions 1:4–1:512 in Minimum Essential Medium (MEM) without fetal calf serum (FCS) were incubated in duplicate with 50 μl of a constant titer of AD169 virus stock (50 immediate early antigen producing units (IEU) per well). After 90 min of incubation at 37°C the virus/serum mixture was added to the monolayer and centrifuged at 1200 × *g* for 30 min. The shell vial culture was allowed to incubate overnight (16–18 hr) in a moist chamber at 37°C with 5% CO_2 in air. Subsequently the cells were fixed for 10 min with ice-cold acetone/methanol (40:60), then blocked with 1% BSA in PBS for 20 min and afterwards were incubated for 30 min with a mixture of murine monoclonal antibodies against 72 kD IE-protein and p52, a 43 kD early-protein diluted 1:100 in PBS (DAKO, Denmark). After washing 3 times with PBS the plates were incubated with a horseradish peroxidase labeled antibody at a dilution of 1:1000 in PBS (anti-mouse-HRP, DAKO, Denmark). The washing step was repeated, HCMV-positive nuclei were stained using the substrate 3-amino-9-ethylcarbazole (AEC) (Sigma, St. Louis, MO) and counted microscopically. The serum dilution producing 50% inhibition of virus infectivity in comparison to an untreated control was reported as the neutralizing titer.

Semi-Automated Avidity Assay

For HCMV-specific IgG avidity, sera were measured by the urea denaturation procedure as described by Hedman et al., [1989] using the Enzygnost-HCMV IgG kit according to the manufacturer's recommendations with minor modification. Each serum sample was diluted as recommended and added to duplicate wells. After the first antibody incubation, one plate was washed with the supplied Enzygnost washing buffer, and in parallel the other plate was washed three times with 6M urea added to the Enzygnost washing buffer. Subsequently, the following steps were carried out on the BEPIII (Dade Behring, Marburg, Germany) according to the manufacturer's recommendations. Control samples of high and low avidity indices were included in each test run. The avidity index was expressed as follows: Percentage of avidity index = (absorbance result of HCMV per well with urea wash/absorbance result of HCMV well without urea wash) × 100. Avidity index <40% indicates acute primary infection, avidity index of 40–60% is considered borderline, and avidity index >60% indicates past infection.

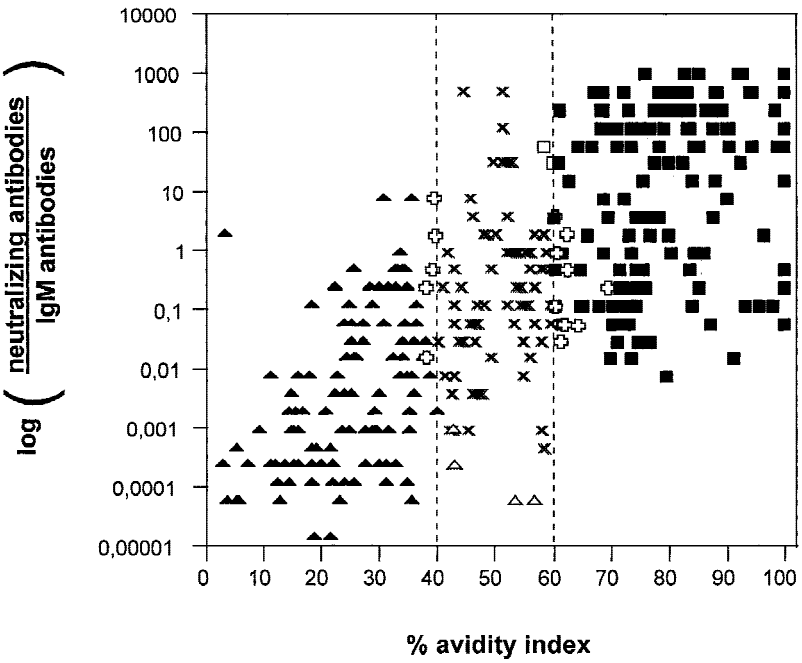

Fig. 1. The ratio of reciprocal neutralization antibody titer to reciprocal IgM-ELA-titer in 350 sera from 227 pregnant women was compared for each single serum sample with the avidity response (% avidity index). Sera with a low positive AI response (<40%) are symbolized by the filled triangles. Serum sample with moderate AI (>40 to <60%) are represented by the cross. The filled square indicates a high avidity response (<60%). These 3 clusters were also compared to the diagnosis based on NT-titer and IgM value. As indicated by the open symbols lack of agreement was only found in a few serum samples. The majority of these nonconsistent sera distributed in the range between low AI and moderate AI or between moderate AI and high AI.

Virological Methods

Neonatal urine obtained 2–3 weeks after birth was tested mainly by the shell vial assay, cell culture and in some cases by PCR as described previously (Daiminger et al., 1994). Fetal tissue obtained after abortion was tested for HCMV using the polymerase chain reaction (PCR), the shell vial assay (detection of HCMV early antigen), and cell culture.

RESULTS

Distribution of avidity indices in primary or nonprimary HCMV infected pregnant women. The avidity indices of HCMV specific IgG antibodies in 227 pregnant women (n = 350 sera) were determined using a semiautomated avidity assay. As shown in Figure 1, the sera were subdivided into 3 clusters. The first group comprises sera with negative or low NT-titers and high IgM titers resulting in a low neutralizing antibodies to IgM antibodies ratio. Most of these serum samples showed low avidity index of <40%. Sera with a high NT-titer and low or negative IgM titer gave avidity indices of more then 60%. The third group could not be readily classified into primary or nonprimary infection because a moderate ratio of reciprocal NT titer to reciprocal IgM-titer and, in addition, a moderate avidity index of >40% to <60% was found. These 3 clusters were also compared to our own diagnosis based on NT-titer and IgM. As indicated by the open symbols in Figure 1, lack of agreement was only found in a few serum samples. The majority of these inconsistent sera distributed in the range between low avidity index and moderate avidity index or between moderate avidity index and high avidity index; however, in the first serum sample at gestational week 30 one pregnant women had a moderate avidity index of 56.6% without having a NT-antibody response that is a reliable marker of acute primary HCMV-infection. The avidity index of blood sample taken one and 11 weeks later remained still moderate (53.1% and 57.8%, respectively), whereas NT seroconversion occurred in gestational week 35. Therefore the avidity index of acutely infected women ranged from 2.8% to 56.6% resulting in a mean value of 25.4%. The mean avidity index of Group III that could not be differentiated in primary or nonprimary HCMV infection was 51.2% (range, 37.7%–69.7%). In addition, the range of avidity values of Group I and Group III showed that the difference between these sera is less distinct when using the avidity index alone. The mean avidity index value of serum samples of previous infected women was 80.4% (range, 58.4%–100%). Relative sensitivity and relative specificity of the avidity test reached 73.5% and 73.6%, respectively, whereas the microneutralization assay had a high relative specificity (100%) and a relative sensitivity of 74.5%. The lowest specificity (56.8%) for the discovery of primary infection was observed when the detection of IgM antibodies was used only (relative sensitivity: 100%) because IgM antibodies persist longer. Positive and negative predictive values of IgM antibod-

TABLE I. Kinetics of the Avidity Maturation of CMV-IgG-Antibodies in Four Representative Women With Well Documented Seroconversion

Case number	Gestation week	Enzygnost anti CMV IgG EIA [U/l] >180 pos	CMV IgM ELA [titer] ≥1:128 pos	ETI-CYTOK-M reverse sorin [index] >1.1 pos	Neutralizing antibodies [titer] ≥1:4 pos	CMV IgG avidity (%) <40% low AI >60% high AI	Outcome of pregnancy/ newborn
1	6	neg	1:32000	8.481	<1:4	ND	not infected
	8	728	1:8192	9.592	<1:4	18.7	
	10	3102	1:4096	8.022	<1:4	22.2	
	14	5294	1:1024	6.989	<1:4	45.1	
	23	4311	1:256	5.321	1:4	49.1	
	27	5370	1:64	4.753	1:8	59.7	
	32	5823	1:1024	10.399	1:64	62.0	
2	30	neg	pos	ND	ND	ND	congenitally infected
	31	327	1:32000	18.045	<1:4	ND	
	34	660	1:4096	8.162	<1:4	30.1	
	pp	1363	1:4096	7.306	1:16	47.8	
	7 weeks pp	3858	1:1024	neg	1:512	58.1	
3	7	neg	neg	ND	ND	ND	not infected
	22	304	1:4096	4.538	<1:4	2.8	
	31	1287	1:1024	borderline	<1:4	35.4	
	36	1590	1:128	neg	1:32	51.2	
	39	2648	1:256	neg	1:32	60.3	
4	10	1514	1:16000	ND	<1:4	12.9	congenitally infected
	12	3026	1:8192	2.024	<1:4	25.6	
	17	9318	1:4096	neg	<1:4	43.1	
	22	7562	1:256	neg	1:32	51.9	
	pp	6428	1:128	neg	1:16	65.3	

AI = avidity index; pp = post partum; ND = not determined.

ies detection, microneutralization assay and avidity test for the detection/exclusion of a primary HCMV infection in pregnancy were examined in 174/350 sera. Unresolved IgM positive sera belonging to Group III were excluded from the calculation. Avidity index and IgM testing with the IgM ELA had a positive predictive value of 74.0% and 47.6%, respectively and a negative predictive value of 100% each. In contrast the microneutralization assay resulted in a positive predictive value of 100% and a negative predictive value of 90.6%.

Kinetics of avidity maturation in pregnant women. The time point of seroconversion was known precisely for four women (Table I). In Cases 1 and 2 an IgG negative, but IgM positive serum sample was obtained, Case 3 developed flu-like symptoms in the 19th/20th week of gestation and in the last case the seroconversion occurred exactly between gestational Weeks 7 and 8. As expected, the neutralizing titers tended to be low after NT seroconversion and were seen to rise in follow-up samples. As shown in Table I, pregnant women undergoing a primary HCMV infection showed initially low avidity indices (<40%) for approximately 10 weeks (range 8–11 weeks) and antibodies reached full maturation after approximately 22 weeks (range 17–30 weeks).

Detection of low avidity-specific IgG antibodies in sera from women with neutralizing antibody response. The next step was to determine the avidity index in 170 sera from 113 pregnant women with suspected primary infection. As demonstrated by 7 representative women in Table II, all of these cases were suspected of being acute primary infections because of low to medium neutralizing antibody titers and a positive IgM-response in at least one of two tests. Definitive evidence as to whether the infection took place before or after conception could not be obtained because sampling occurred later in pregnancy. In Cases 5 and 6 the low positive NT titer and high positive IgM response in both tests was highly suggestive of a recent primary infection. This was confirmed by the low positive avidity result. The serological data of Case 7 were not highly suggestive of an acute infection except for the low avidity index of 33.1%. All 3 newborns proved to be congenitally infected because HCMV was detectable in the urine samples collected within the first two weeks of life. In contrast, pregnant women having a high avidity index response (Table II, Cases 8–11) seem to be at no risk of giving birth to a congenitally infected child.

Outcome of pregnancy. Transmission of HCMV to the fetus occurs in approximately 40% of primary HCMV infected women whereas approximately 0.5% of nonprimary maternal infections leads to an intrauterine HCMV transmission. To differentiate between primary and nonprimary maternal infection for estimation of the putative risk of an intra-uterine HCMV transmission to the fetus either the microneutralization test alone or the combined application of microneutralization test and avidity assay was used (Table III). The outcome of pregnancy was known for 30 of the tested women. In all cases primaryinfection was suspected by the above mentioned criteria. For diagnosis of congenital infection virus was detected in urine

TABLE II. Seven Representative Cases With Serologically Suspected Primary Infection in Pregnancy*

Case number	Gestation week	Enzygnost anti CMV IgG EIA [U/l] >180 pos	CMV IgM ELA [titer] ≥1:128 pos	ETI-CYTOK-M reverse sorin [index]	Neutralizing antibodies [titer] ≥1:4 pos	CMV IgG avidity (%) <40% low AI >60% high AI	Outcome of pregnancy/ newborn
5	27	1892	1:4096	5.538	1:4	16.1	congenitally infected
	29	2346	1:4096	7.680	1:64	26.0	
	pp	2572	1:256	1.706	1:512	48.6	
6	9	3404	1:8192	2.965	1:16	20.8	congenitally infected
	12	4538	1:4096	2.392	1:16	25.2	
	18	4840	1:1024	1.628	1:512	34.1	
	21	4689	1:4096	1.589	1:512	42.7	
	24	5823	1:1024	1.368	1:512	75.6	
7	18	2950	1:128	1.063	1:64	33.1	congenitally infected
	22	4160	neg	neg	1:64	48.8	
	23	2724	neg	neg	1:64	56.6	
	24	2192	neg	neg	1:128	51.8	
	pp	2194	neg	ND	1:128	68.3	
8	8	1514	1:512	1.476	1:32	56.3	not infected
	12	1287	1:512	1.256	1:8	59.4	
	15	1590	1:256	1.406	1:32	64.5	
	34	1134	1:512	1.561	1:16	55.7	
	pp	1287	1:512	ND	1:16	55.4	
9	31	6806	1:4096	borderline	1:256	99.8	not infected
10	22	10586	1:128	borderline	1:128	85.9	not infected
11	21	3328	1:128	neg	1:512	78.4	not infected
	22	3328	1:256	neg	1:256	84.2	

*AI = avidity index; pp = post partum; ND = not determined.

TABLE III. Estimation of the Risk of Intra-Uterine Transmission of CMV to the Fetus in 30 Women With Suspicious Serology (IgM Antibody Titer Positive)

Outcome of pregnancy/newborn	Primary infection during pregnancy	Unable to be differentiated by serological methods	Nonprimary infection during pregnancy
Differentiation into subgroups by using the microneutralization assay			
Infected	7	5	0
Not infected	7	11	0
Total (n = 30)	14	16	0
Differentiation into subgroups by using the combined application of microneutralization assay and avidity test			
Infected	12	0	0
Not infected	13	2	3
Total (n = 30)	25	2	3

samples within the first two weeks of life by the rapid shell vial assay. In total, 12 out of the 30 investigated neonates were infected congenitally resulting in a transmission rate of 40%, however none had clinical symptoms at birth.

Using solely the NT assay, 14 of 30 women were categorized as primary infection during pregnancy. The diagnosis was based on the absence of neutralizing antibodies in the first serum sample. Seven of the 14 newborns of this group were infected with HCMV (50%). None of the 30 investigated women belonged to the nonprimary HCMV-infection group. Sixteen of the investigated neonates were born to mothers whose serostatus could not be readily differentiated in primary or nonprimary infected by the microneutralization test; however, 5 out of those 16 neonates from women with unclear immune status were infected asymptomatically (31%).

If the diagnosis was based on the combined application of microneutralization test and avidity index, only 2 cases with a low avidity response in follow up could not be serologically differentiated in primary or nonprimary infected (see Table III). Both women gave birth to uninfected babies. In addition, 3 women who were all nontransmitters could be identified as belonging to the nonprimary infected category due to high avidity indices. All 25 women with low avidity indices belonged to the primary infected group. Congenital HCMV infection was confirmed in 12 out of those 25 pregnancy outcomes (48%). Using the microneutralization assay alone, 7 of the 12 transmitting mothers (58.3%) were identified readily as primary infected; however the combined application of avidity test and microneutralization assay leads to the recognition of all 12 pregnancies with HCMV transmission (100%).

DISCUSSION

The diagnosis of HCMV infection acquired recently in pregnant women should be made as early as possible in pregnancy to identify those women who are at risk of transmitting the virus to the fetus and for advice on further management [Enders 1998; Lazzarotto et al., 1998]. The aim of HCMV serology is to avoid unnecessary prenatal diagnosis in women with long persisting IgM by differentiating between primary and nonprimary HCMV-infection. We have demonstrated recently [Eggers et al., 1998] that if the HCMV microneutralization assay is carried out very early in pregnancy it can identify all pregnant women who will transmit HCMV to their offspring. Unfortunately, many countries including Germany do not screen for HCMV antibodies during routine antenatal care programs or even estimate the preconceptional serostatus. This poses a risk of being unaware of congenital infection in a newborn. Infants and toddlers born with congenital HCMV infection need frequent neurodevelopemental and physical examinations by pediatricians because in the early months and years high-severity disabilities, such as cerebral palsy and severe mental retardation may be discovered. Over 10% of the infants with asymptomatic congenital HCMV infection and two-thirds of children with symptomatic congenital HCMV infection will develop progressive sensorineural hearing loss or other neurodevelopemental difficulties within the first 4 years of life [Williamson et al., 1992; Demmler 1994; Fowler et al., 1997]. Laboratories receive frequently blood samples at a late stage of pregnancy due to suspicious influenza-like symptoms where diagnosis of an acute primary infection is no longer possible. In second trimester serum samples it may be difficult to date a recent maternal primary infection using only the microneutralization test particularly if medium to high neutralizing antibody titers and low positive IgM antibody values are detectable as seen in Case 7 of Table II. Therefore, an alternative approach is necessary to ensure the identification of all congenital HCMV infections. Another serological method for identifying primary infection is the determination of IgG avidity [Blackburn et al., 1991; Lazzarotto et al., 1997; Grangeot-Keros et al., 1997; Bodeus et al., 1998; Bodeus and Goubau, 1999; Lazzarotto et al.,1999]. One of the drawbacks of this assay, however, is that for calculation of the avidity index a minimum IgG concentration of 1000 PEI U/l is necessary. The IgG value of six of the investigated 350 sera (1.6%) fell below this detection limit and the avidity index could not be calculated on the OD values (data not shown). In addition we observed a lack of reproducibility in low avidity serum samples. For investigation of the intra-assay and inter-assay reproducibility three different pool sera prepared from samples with low (mean avidity index of 31.5%), medium (mean avidity index of 60.3%) and high avidity (mean avidity index of 80.6%) antibodies against HCMV were assayed 7-fold on 5 different days. The results showed the reproducibility of the high and moderate avidity sera to be good with CVs of 8.8% and 12.2%, respectively, whereas the CV was 21.8% for the low avidity pool. For control of the assay reproducibility suitable controls with low avidity index, moderate avidity index and high avidity index have to be included in each test run. As mentioned by B. Weißsbrich [1998] automation is also important to standardize the measurement of IgG avidity and therefore to increase the acceptance of avidity assays in routine laboratories. In accordance with his recent study of EBV IgG avidity determination we used a semi-automated avidity method in which all processing steps of the microplates except for the urea washing were carried out automatically.

In this study a moderate avidity index was observed in the first serum sample of a pregnant woman even when no neutralizing antibodies were detectable. This woman transmitted the virus to her offspring. And on the other hand in 2 of 137 investigated women with low positive IgM values a low avidity response in follow up could be detected (Table III). Both women who had neutralizing antibodies in the first serum sample gave birth to an uninfected healthy baby. For that reason an acute primary infection in pregnancy according to the proposed serological approach of combined application of microneutralization assay and avidity test is definitely proven by lack of neutralizing antibodies. In case of a low neutralizing titer and positive IgM value the avidity test should be undertaken. A low avidity index is highly suggestive of an acute primary infection in pregnancy. In consecutive serum samples of pregnant women with well-documented seroconversion (Table I) the antibody response had matured to be of high avidity within an average of 22 weeks (17–30 weeks). This observation is in accordance to the data of Lazzarotto et al., [1997] who reported the appearance of high avidity index in transplant recipients after approximately 25 weeks and in pregnant women after 18–20 weeks. As presented in Table III with the combined application of the microneutralization assay and avidity assay all women who gave birth to congenitally infected infants were shown to have a primary infection. Pregnant women with serologically suspected primary infection during the first two trimesters prenatal diagnosis including high level ultrasonography mainly between gestational week 19–24 should be offered (Enders, 1998).

The overall transmission rate in the investigated primary infected group having low avidity index was 48% that is consistent with the findings of several authors who reports a mean rate of 40% [Stagno et al., 1986; Demmler, 1991].

The combined application of microneutralization assay and avidity test is a helpful tool for the diagnosis of a recent primary HCMV infection in second trimester pregnancy particularly in the absence of previous serological data.

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